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14. ABSTRACT
Prostate cancer has been predicted to cause nearly 10% of male cancer deaths in US in 2007. The frequency of mutations in the tumor suppressor genes (pRb and p53) is rare in prostate cancer. This has led to the possibility that a human virus like BK virus (BKV), which establishes a lifelong, subclinical persistent infection in the urinary tract and encodes oncoproteins (large T antigen, TAg; small T antigen, tAg), which interfere with these tumor suppressor pathways, may play a role in the early stages of the disease. Our initial analysis of cancerous prostate tissues shows the presence of BKV in normal and atrophic epithelium, which is a precursor lesion to prostate cancer and metastatic disease. TAg, which is a nuclear protein, is expressed in the atrophic cells, is cytoplasmic, and co-localizes with p53. We have extended our analysis to show that BKV is present at a much lower frequency in non-cancerous prostate. Additionally, TAg expression in normal prostate is only observed in specimens that have proliferative inflammatory atrophy and prostatic intraepithelial neoplasia. Utilizing laser capture microdissection, we observe an inverse correlation between TAg expression in atrophic epithelium and mutations in the p53 gene in those cells. This supports our hypothesis that BKV inactivates p53 in the early stages of prostate cancer. Our data suggests there could be a potential link between BKV and prostate cancer.

15. SUBJECT TERMS
BK virus, Large T antigen, atrophic lesions, p53, laser capture microdissection, nuclear localization signal

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INTRODUCTION:

Prostate Cancer (PCa) has been projected to be the leading cause of cancer related deaths in older men by the year 2010 (12). An increased understanding of basic molecular mechanisms that initiate prostate cancer would be very useful for the development of treatment strategies and early detection. Recently, evidence in support of proliferative inflammatory atrophy (PIA) as the potential precursor of prostate adenocarcinoma has been accumulating. Prostatic atrophy is being proposed as the stage that changes benign epithelium to adenocarcinoma, due to the proliferative nature of the atrophic lesions, when compared to normal epithelial cells (reviewed in 4).

In normal prostate, the secretory ducts are lined by two layers of epithelial cells, the basal layer and second layer of luminal cells that are columnar. With disease progression, the normal epithelium transitions to PIA. In this stage, the basal epithelial cell layer is lost and the luminal secretory cells lose much of their cytoplasmic mass. The next stage in the PCa progression pathway is prostatic intraepithelial neoplasia (PIN), where the cells acquire an abnormal nuclear morphology and appear irregular. PIN changes over to localized cancer where the dual epithelial layer of cells is lost, cells have a higher nuclear to cytoplasmic ratio with prominent nucleoli. The final stage of the disease is the metastatic state (reviewed in 4). It is interesting to note here that both the tumor suppressor genes (*p53 and Rb1*), are lost in the later stages of prostate cancer. Additionally, the occurrence of mutations in tumor suppressor genes (*p53 and Rb1*) in the early stages of prostate cancer is relatively low. This has lead to the speculation that a viral agent which interferes in these tumor suppressor pathways may play a role in the early stages of prostate cancer.

BK virus (BKV), a member of the polyomavirus family is an excellent candidate for this agent. It is ubiquitous in the human population and infects about 100% of the population by early childhood. It establishes a lifelong subclinical persistent infection in the urinary tract and upon immunosuppression, particularly in renal and bone marrow transplant patients, can be reactivated and cause severe disease in the kidneys and bladder, respectively. BKV oncogenically transforms rodent and primary human cells in culture and causes tumors in transgenic mice. Its co-expression with an activated *ras* oncogene leads to the transformation of primary human cells in culture. Oncogenesis is mediated by the expression of the large and small tumor antigens (TAg and tAg). TAg expression induces oncogenic transformation through the inactivation of the two tumor suppressor pathways (p53 and RB) (reviewed in 9, reviewed in 27). This inactivation is essential for efficient viral replication and cellular transformation. This is very similar to how E6 and E7 gene products of human papillomavirus (HPV) inactivate p53 and RB, in cervical carcinomas (2, 10, 25). Wild type p53 levels are usually low in a cell whereas the mutant form of p53 is more stable and occurs at a higher frequency. Viral oncoproteins inactivate p53 by binding to it and sequestering the protein in an inert form (18, 28). So, upon polyomavirus infection, wild type p53 levels are elevated in response to TAg expression but p53 is functionally inactivated due to its sequestration by TAg (17, 19, 22-24).

Our preliminary analysis of cancerous prostate tissues obtained from radical prostectomies shows the presence of BKV DNA sequences in the epithelial cells of benign and atrophic ducts, which is the initial step in prostate cancer and, TAg expression is specifically localized to atrophic epithelial cells. TAg is cytoplasmic, indicating that viral replication cannot be occurring. Additional staining for VP1, which is a replication marker for BKV, is also negative. TAg also co localizes with p53 implying that p53 is nonfunctional (3).

BKV is not observable in the later stages of cancer, implying it is lost as the cancer advances to PIN and the metastatic stage.

We have extended our preliminary studies to further determine if BKV plays a possible role in early stages of prostate cancer. Our hypothesis is that TAg inactivates p53 in the early stages of prostate cancer. Our hypothesis of the status of *p53* gene in BKV-related prostate cancer is derived from the observation in cervical carcinomas. HPV induced cancers contain wild type p53 while, HPV negative cancers have mutant p53 (2, 10, 25). We predict that in BKV TAg expressing cells with cytoplasmic p53, wild type *p53* gene will be observed and in BKV TAg-negative tumor cells with detectable nuclear p53, mutant *p53* gene will be detected.

There are two specific aims of this study. The first specific aim was to utilize laser capture microdissection to analyze the sequence of *p53* gene in T antigen-positive and negative cells. This will also include the analysis of both normal and diseased prostate for the presence and expression of BKV. The second specific aim was to characterize BKV tissue isolates by investigating the status of replication properties of BKV using genomic clone constructions derived from patient sequences and then monitoring replication. The first specific aim (Task 1) of the statement of work was fully completed and has been submitted to *J. Virol*. Due to time constraint, the second specific aim could not be completed.

BODY:

Aim1: To determine the status of *p53* gene in BKV TAg positive and negative glands in prostate tissues by using laser capture microdissection (LCM) technique to extract DNA followed by PCR amplification.

For this analysis, we first designed PCR primers to amplify exons 5-9 of the *p53* gene. These exons are the sites for the most frequent mutations in the *p53* gene in various cancers (8, 16, 21). Next, we optimized the primers for amplification from 200 to 1000 number of cells. The primers were first used to amplify DNA extracts prepared from whole tissue sections that were either positive for TAg expression or, negative for TAg expression but expressed nuclear p53. Whole prostate tissue sections contain a mixture of TAg-positive atrophic cells and TAg-negative tumor, normal and stromal cells. DNA extraction was performed as described previously (3). LCM system was also used to dissect specific areas of the paraffin embedded prostate tissue sections that either, express cytoplasmic TAg (atrophic, PIA cells) or nuclear p53 (tumor cells) (5, 7). Figure 1 (A/B) shows LCM captured cells from an immunostained prostate tissue section. After immunohistochemistry (IHC) with the respective antibodies, prostate tissue sections were exhaustively dehydrated in ethanol and air dried before laser capture microdissection (6). Laser capture was performed using the PIXCELL II Laser Capture Microscope. Immunostained cells from 5 µM tissue sections, obtained from an average of 1500 firings of a 7.5 uM laser spot size, was captured on the thermoplastic caps and digested in 20 uL proteinase K buffer. After the inactivation of proteinase K, DNA extracts were amplified with p53 specific primers to exon 5-9 and sequenced.

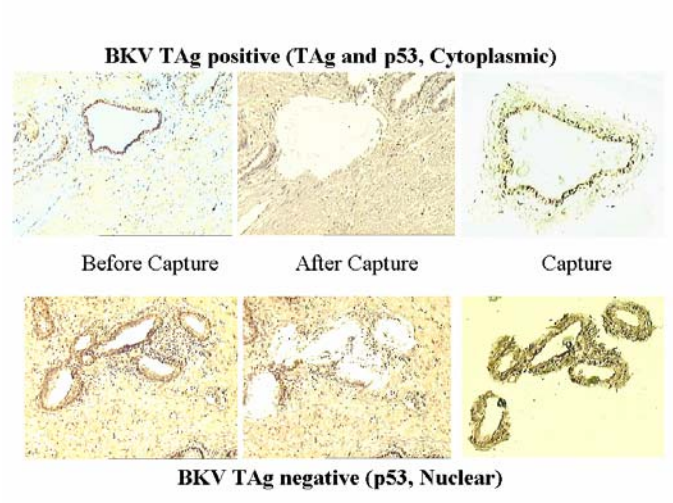

Figure 1. LCM of TAg positive atrophic cells and TAg negative tumor cells expressing nuclear p53. Before and after capture of the glands is shown in these panels. Panel **A** is a micrograph of a TAg positive sample that expresses cytoplasmic TAg in atrophic glands. Panel **B** is also a micrograph of a TAg negative sample that expresses nuclear p53.

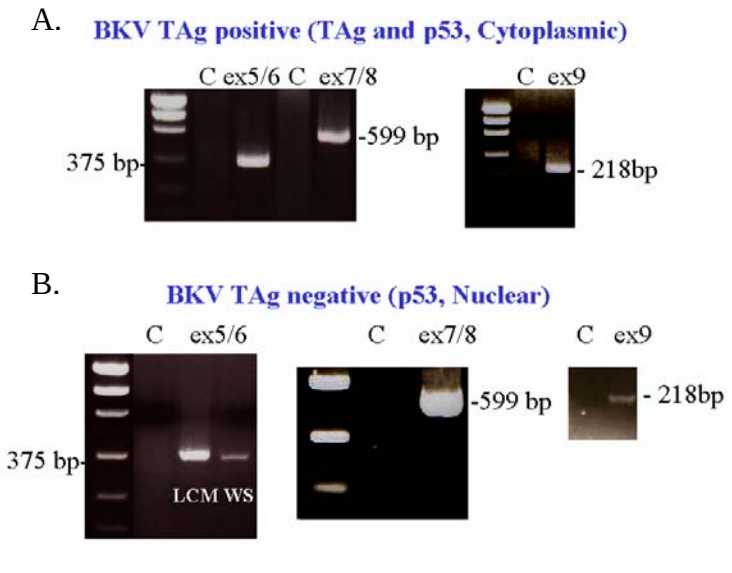

Figure 2. DNA Amplification of the exon 5-9 of p53 gene from laser captured cells. Panel **A** represents amplification from cytoplasmic TAg positive atrophic cells and panel **B** is amplification from TAg negative tumor cells expressing nuclear p53. C is control from a reaction using water as the template. WS is amplification from whole section which contains a mix of tumor, normal, atrophic and stromal cells.

Chromatograms were carefully examined for the presence or absence of mutations. An example of the chromatogram is shown in figure 3.

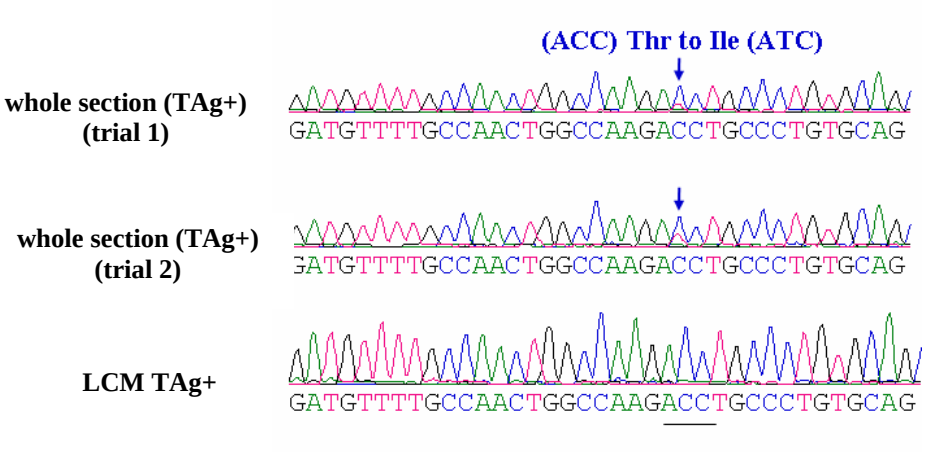

Figure 3. Chromatogram of the p53 sequence. Partial sequences of Sample1 from Table 1 are shown. Arrows show a mix of wildtype (cytosine) and mutant (thymidine) form of p53, which translates to amino acid 140 in the whole section DNA. Careful analysis of the sequence shows both nucleotides even though the software recognized that position as a cytosine. The line represents the wild type (cytosine) form at the same position which is from laser captured TAg+ cells.

To date, all the TAg positive LCM samples express wild type p53, whereas some of the TAg positive samples from entire thin sections contain a mix of wild type/mutant p53. It is also important to mention here that all the intron mutations observed so far in this study has been previously reported (1). Whole tissue sections contain a mix of tumor, normal, atrophic and stromal cells and it is possible that the mutant p53 sequence that we observe from TAg positive whole sections is the amplification of p53 from tumor cells. One of the LCM sample (sample 4) that is TAg negative but expresses nuclear p53 contains a mix of wild type/mutant p53. This may be either due to the contamination of the LCM cells (tumor cells; nuclear p53) with the stromal cells or due to the heterozygosity at the locus. The interesting point to note here is that p53 sequence of pure population of TAg expressing atrophic cells obtained by LCM is wild type whereas LCM obtained tumor cells expressing nuclear p53 harbor mutations in the p53 gene. This supports our hypothesis that TAg inactivates p53 in the early

stage of prostate cancer. The sequence analysis of the *p53* gene obtained to date is shown in Table 1.

It is also interesting to note here that TAg is not detectable in the later stages of prostate cancer and p53 is also lost as cancer progresses from localized to metastatic stage. It is tempting to speculate here that in the set of samples that are positive for BKV TAg expression, p53 is being inactivated at an early stage in the PCa pathway.

Table 1

Sequence Analysis of the *p53* Gene

Sample	Exons sequenced	sequence, whole section	sequence, [A]LCM
1	5-6	wt, mt (Thr 140 to Ile)	wt
	9	Wt	wt
2	5-6	Wt	wt
	7-8	wt, mt[B, C]	wt
	9	Wt	wt
3	5-6	wt	wt
	7-8	wt.mt[C]	ND
	9	Wt	wt
7	5-6	mt (Pro 191 to Leu)	wt
	9	wt, mt[C]	wt
8	9	wt	wt
4	5-6	wt	wt
	7-8	wt, mt[C]	wt
	9	Wt	wt, mt (Pro318toSer)
5	5-6	wt	wt
	9	Wt	wt
6	5-6	wt	wt
	7-8	wt, mt[C]	ND
	9	Wt	wt

[A] Laser captured cells, Samples 1, 2, 3, 7, 8 were TAg-positive, cytoplasmic p53; Samples 4-6 were TAg-negative, nuclear p53

[B] Previously reported intron mutation in A549 lung carcinoma cell line (31)

[C] Previously reported intron mutations listed in IARC TP53 mutation database (32); IARC is the International Agency for Research on Cancer

ND, sequence not determined

Furthermore, we also tried RT-PCR from formalin fixed paraffin embedded whole prostate tissue sections. The RT-PCR sequence analysis of the *p53* gene was not very reproducible whereas the DNA sequence analysis was very consistent. Reported DNA mutations in the *p53* gene could be amplified from the prostate tissue sections. So, all our p53 analysis is based on DNA amplification.

Aim 2: Comparison of cancerous prostate with normal prostate.

The second aim of the project was to determine if BKV resides naturally in the prostate since it is ubiquitously present in the population or if the diseased tissues have a higher incidence of BKV. A cancerous prostate is defined as prostate that has been surgically removed due to prostate cancer diagnosis. A normal prostate is defined as prostate that has been removed either during autopsy or by cystoprostatectomy from a bladder cancer patient who has no histological signs of prostate cancer. Normal prostates were analyzed for BKV using *in situ* hybridization (ISH) and immunohistochemistry (IHC). BKV DNA was present at a much lower frequency in normal prostate than in cancerous prostate. In the majority of normal samples tested to date, BKV DNA was absent. However in a small set of normal samples, BKV DNA was observed both in the normal epithelium and atrophic cells (Figure 4 and Table 2). Similarly, in a very small percent of the normal samples, TAg was in the atrophic epithelium and was cytoplasmic. Additionally, the same TAg positive samples were also positive for p53, and it was also cytoplasmic. There was no expression of TAg in normal epithelium. This study shows that BKV is present at a significantly higher frequency in cancerous prostate and TAg is being specifically expressed in the

atrophic epithelium of normal prostates. Fisher's exact test both for ISH (p = 0.007) and IHC (p = 0.008) shows significant difference between the BKV positive samples in normal and cancerous prostates (Table 2). This suggests that BKV does not reside naturally in the prostate epithelium. The presence of BKV sequences and expression of BKVTAg in PIA lesions also suggests that BKV may cause the transition of the tissues from benign to atrophic, ultimately leading to PCa.

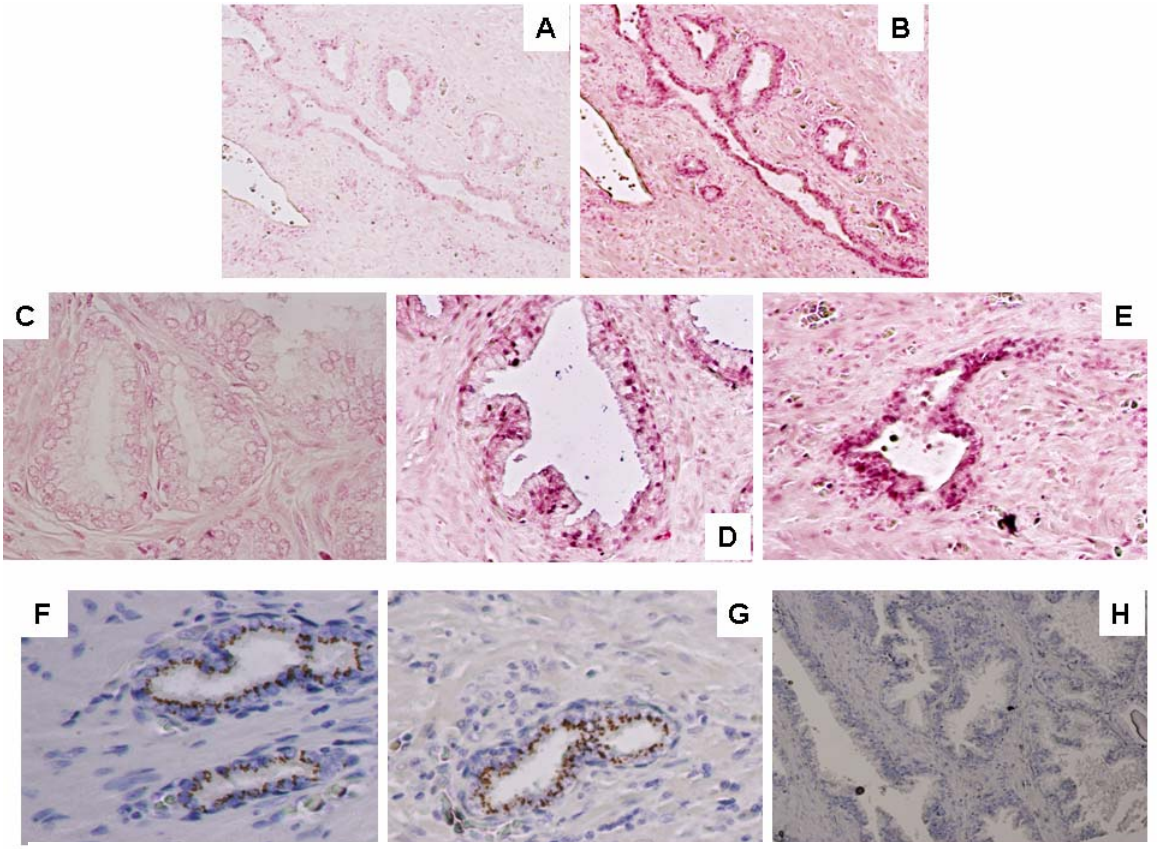

Figure 4. *In situ* hybridization and immunohistochemistry of normal prostates. A and **B** are serial sections hybridized with scrambled and BKV-specific probe, respectively. **C** is a normal prostate sample that is negative for BKV DNA, whereas **D** and **E** are normal and atrophic ducts, respectively of the sample showing positive staining with BKV-specific probe. **F** and **G** are the same samples immunostained with anti TAg and anti p53, respectively, showing cytoplasmic expression in the atrophic ducts. **H** is a normal gland that is negative for TAg expression.

Table 2

Comparison of BKV in normal and cancerous prostate

	Normal	Cancer	*p*-value
BKV DNA +	4	11	
BKV DNA -	11	3	0.007
TAg +	4	13	
TAg -	25	15	0.008

+, positive; -, negative; BKV DNA, ISH was done; TAg, IHC was done

We had also planned to extend this analysis to prostate cancer tissue microarrays, to increase sample size but we are unable to use TMAs for our analysis because PIA lesions are underrepresented in these prostate cancer TMAs. So, we cannot analyze large number of samples at this time.

Aim 3: NLS analysis of TAg of BKV

The third aim of the project was to determine the integrity of the nuclear localization signal (NLS) of BKV TAg since it is specifically expressed in the cytoplasm rather than its normal nuclear site. So, primers were designed to amplify the NLS of TAg which is KKKRK (13, 15). TAg was amplified from three TAg positive prostate samples. Chromatogram is shown in figure 5. Wild type NLS was observed in all the three samples that were sequenced (Table 3).

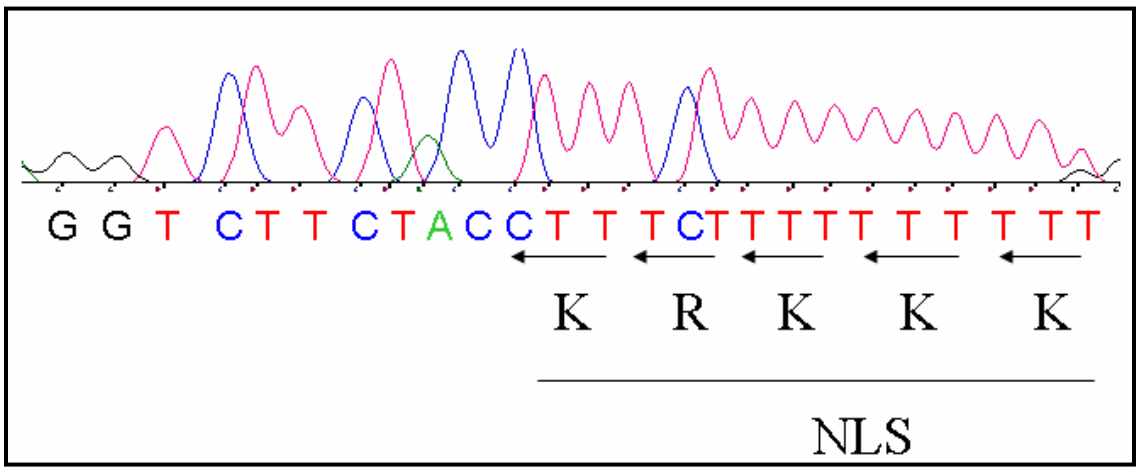

Figure 5. Chromatogram of the nuclear localization signal (NLS) of BKV TAg. Partial sequence of BKV TAg from cancerous prostate expressing cytoplasmic TAg is shown. The NLS sequence is wild type.

Table 3

NLS Sequence Analysis of BKV TAg

Sample (cancer)	TAg+	p53+	NLS (TAg)
1	Cyto	Cyto	wt
2	Cyto	Cyto	wt
3	Cyto	Cyto	wt

Cyto, cytoplasmic expression; wt, wild type

This implies that some cellular factor is responsible for sequestering TAg, and in consequence p53, in the cytoplasm. It has been reported that nuclear import of TAg proteins is inhibited by p34cdc2-mediated phosphorylation of TAg at specific threonine residues (11, 26). Complex formation between p53 and p34cdc2 has been reported and TAg stimulates expression of cdc2 gene (20).

There are also reports of p34cdc2 expression in prostate cancer and increased levels have been shown to associate with disease progression (14).

KEY RESEARCH ACCOMPLISHMENTS:

The key accomplishments to date are as follows:

- Data obtained to date from the LCM analysis of p53 gene from TAg positive and negative prostate cancer samples supports our hypothesis that BKV inactivates p53 in the early stage of prostate cancer in the subset of samples that are positive for TAg expression. If a correlation can be established between p53 and TAg in the early stage prostate cancer, then antiviral inhibitors can be designed to target BKV TAg.
- Comparative analysis to date of normal prostate with cancerous prostate for BKV indicates that BKV is present at a significantly higher frequency in cancerous prostates than in normal prostates. This suggests that BKV may be involved in the early stage of prostate cancer progression.

REPORTABLE OUTCOMES:

Dweepanita Das, Rajal B Shah and Michael J. Imperiale. The Role of BK Virus in the Early Stages of Prostate Cancer. DNA Tumor Viruses 2006 Meeting. Oral Presentation. Salk Institute, La Jolla, CA. July 11-16, 2006.

Dweepanita Das, Kirk Wojno and Michael J. Imperiale. Role of Human Polyomavirus BKV in Prostate Cancer. The Innovative Minds in Prostate Cancer Today (IMPACT) September 5-8, 2007, Hyatt Regency, Atlanta

Dweepanita Das, Kirk Wojno and Michael J. Imperiale. BKV as a Cofactor in the Etiology of Prostate Cancer in its Early Stages. Submitted to *J. Virol.*

CONCLUSIONS:

BKV is present at a significantly higher frequency in cancerous prostates. It does not reside naturally in the normal prostate. The percentage of normal prostates harboring BKV is significantly low. This strongly supports a connection between BKV and cancer. TAg expression is significantly lower in normal prostates and is specifically localized to atrophic epithelium suggesting that these individuals may be predisposed for prostate cancer in later years or if lived longer (autopsy samples). We observe an inverse correlation between TAg expression in atrophic epithelium and mutations in the *p53* gene in those cells. This supports our hypothesis that BKV TAg inactivates p53 in the early stage of prostate cancer. Additionally, the NLS sequence of TAg expressed in prostate samples is intact, implying that a cellular factor is sequestering TAg, and as a result p53, in the cytoplasm.

The above studies are intriguing and suggest that BKV may play a role in the etiology of prostate cancer. If a link between BKV and prostate cancer is established, its unique viral properties can be used for the possibility of vaccination for prevention or treatment by designing drugs that target TAg.

REFERENCES:

1. **Anderson, C. W., and M. J. Allalunis-Turner.** 2000. Human TP53 from the Malignant Glioma-Derived Cell Lines M059J and M059K Has a Cancer-Associated Mutation in Exon 8. Radiaton Research **154:** 473-476.
2. **Choo, K. B., and K. Y. Chong.** 1993. Absence of mutation in the p53 and the retinoblastoma susceptibility genes in primary cervical carcinoma. Virology **193:**1042-1046.

3. **Das, D., R. B. Shah, and M. J. Imperiale.** 2004. Detection and expression of human BK virus sequences in neoplastic prostate tissues. Oncogene **23:**7031-7046.
4. **De Marzo, A. M., E. A. Platz, S. Sutcliffe, J. Xu, H. Gronberg, C. G. Drake, Y. Nakai, W. B. Isaacs, and W. G. Nelson.** 2007. Inflammation in prostate carcinogenesis. Nat Rev Cancer **7:**256-269.
5. **Emmert-Buck, M. R., R. F. Bonner, P. D. Smith, R. F. Chuaqui, Z. Zhuang, S. R. Goldstein, R. A. Weiss, and L. A. Liotta.** 1996. Laser Capture Microdissection. Science **274:**998-1001.
6. **Enam, S., L. Del Valle, C. Lara, D. Gan, H. Ortiz, P. Carlos, Juan P., and K. Khalili.** 2002. Association of Human Polyomavirus JCV with Colon Cancer: Evidence for Interaction of Viral T-Antigen and {beta}-Catenin. Cancer Res. **62:**7093-7101.
7. **Fend, F., M. Kremer, and L. Quintanilla-Martinez.** 2000. Laser Capture Microdissection: Methodical Aspects and Applications with Emphasis on Immuno-Laser Capture. Microdissection. Pathobiology **68:**209-214.
8. **Hollstein, M., D. Sidransky, B. Vogelstein, and C. C. Harris.** 1991. p53 mutations in human cancers. Science **253:**49-53.
9. **Imperiale, M. J.** 2001. Oncogenic transformation by the human polyomaviruses. Oncogene **20:**7917-7923.
10. **Iwasaka, T., M. Oh-uchida, N. Matsuo, M. Yokoyama, K. Fukuda, K. Hara, K. Fukuyama, K. Hori, and H. Sugimori.** 1993. Correlation between HPV positivity and state of the p53 gene in cervical carcinoma cell lines. Gynecol. Oncol. **48:**104-109.
11. **Jans, D. A., M. J. Ackermann, J. R. Bischoff, D. H. Beach, and R. Peters.** 1991. p34cdc2-mediated phosphorylation at T124 inhibits nuclear import of SV-40 T antigen proteins. J Cell Biol **115:**1203-1212.
12. **Jemal, A., R. Siegel, E. Ward, T. Murray, J. Xu, and M. J. Thun.** 2007. Cancer Statistics, 2007. CA Cancer J Clin **57:**43-66.
13. **Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith.** 1984. A short amino acid sequence able to specify nuclear location. Cell **39:**499-509.
14. **Kallakury, B. V., C. E. Sheehan, R. A. Ambros, H. A. Fisher, J. Kaufman, R. P., and J. S. Ross.** 1997. The prognostic significance of p34cdc2 and cyclin D1 protein expression in prostate adenocarcinoma. Cancer Res. **80:** 753-763.
15. **Lanford, R. E., and J. S. Butel.** 1984. Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. Cell **37:**801-813.
16. **Levine, A. J., J. Momand, and C. A. Finlay.** 1991. The p53 tumour suppressor gene. Nature **351:**453-456.
17. **Lin, J. Y., and D. T. Simmons.** 1990. Transformation by simian virus 40 does not involve the mutational activation of p53 to an oncogenic form. Virology. **176:**302-305.
18. **Liu, X., and R. Marmorstein.** 2006. When viral oncoprotein meets tumor suppressor: a structural view. Genes Dev. **20:**2332-2337.
19. **Mietz, J. A., T. Unger, J. M. Huibregtse, and P. M. Howley.** 1992. The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. EMBO J. **11:**5013-5020.
20. **Milner, J., A. Cook, and J. Mason.** 1990. p53 is associated with p34cdc2 in transformed cells. EMBO J. **9:**2885-2889.
21. **Navone, N. M., P. Troncoso, L. L. Pisters, T. L. Goodrow, J. L. Palmer, W. W. Nichols, A. C. von Eschenbach, and C. J. Conti.** 1993. p53 protein accumulation and

gene mutation in the progression of human prostate carcinoma. J Natl Cancer Inst. **85:**1657-1669.

22. **Oren, M., W. Maltzman, and A. J. Levine.** 1981. Post-translational regulation of the 54K cellular tumor antigen in normal and transformed cells. Mol. Cell. Biol. **1:**101-110.
23. **Reich, N. C., M. Oren, and A. J. Levine.** 1983. Two distinct mechanisms regulate the levels of a cellular tumor antigen, p53. Mol. Cell. Biol. **3:**2143-2150.
24. **Reihsaus, E., M. Kohler, S. Kraiss, M. Oren, and M. Montenarh.** 1990. Regulation of the level of the oncoprotein p53 in non-transformed and transformed cells. Oncogene **5:**137-145.
25. **Scheffner, M., K. Munger, J. C. Byrne, and P. M. Howley.** 1991. The state of the p53 and retinoblastoma genes in cervical carcinoma cell lines. Proc. Natl. Acad. Sci. USA. **88:**5523-5527.
26. **Scheidtmann, K. H., J. Schickedanz, G. Walter, R. E. Lanford, and B. J. S.** 1984. Differential phosphorylation of cytoplasmic and nuclear variants of simian virus 40 large T antigen encoded by simian virus 40-adenovirus 7 hybrid viruses. J Virol **50:**636-640.
27. **Tognon, M., A. Corallini, F. Martini, M. Negrini, and G. Barbanti-Brodano.** 2003. Oncogene transformation by BK virus and association with human tumors. Oncogene **22:**5192-5200.
28. **Vousden, K. H., and C. Prives.** 2005. P53 and prognosis: New insights and further complexity. Cell **120:**7-10.

Paper Submitted to J. Virol. is attached in the appendix

BKV as a Cofactor in the Etiology of Prostate Cancer in its Early Stages

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Keywords used are: BKV; large T antigen; p53; proliferative inflammatory atrophy

ABSTRACT

Prostate cancer has been projected to cause almost 10% of all male cancer deaths in 2007 in the US. The incidence of mutations in the tumor suppressor genes, Rb1 and p53, especially in the early stages of the disease, is low when compared to other cancers. This has led to the hypothesis that a human virus such as BK virus (BKV), which establishes a subclinical persistent infection in the urinary tract and encodes oncoproteins that interfere with these tumor suppressor pathways, is involved. Previously, we detected BKV DNA in the epithelial cells of benign and proliferative inflammatory atrophy ducts of cancerous prostate specimens. In the present report, we demonstrate that BKV is present at a much lower frequency in non-cancerous prostates. Additionally, in normal prostates, TAg expression is only observed in specimens harboring proliferative inflammatory atrophy and prostatic intraepithelial neoplasia. We further demonstrate that the p53 gene from atrophic cells expressing TAg is wild type, whereas tumor cells expressing detectable nuclear p53 contain a mix of wild type and mutant p53 genes, suggesting that TAg may inactivate p53 in the atrophic cells. Our results point towards a role for BKV in early prostate cancer progression.

INTRODUCTION

Prostate cancer is the leading cause of male cancer deaths in the United States. The latest statistics for 2007 estimate 218,890 new prostate cancer cases and 27,050 deaths (39). Prostate cancer is a slowly progressing disease; therefore, identification of its precursor lesions has become the primary focus of many studies (reviewed in 14, 20, 30, 55). An increased understanding of the molecular mechanisms associated with prostate cancer initiation and progression would be useful for developing strategies for its early detection and treatment.

Proliferative inflammatory atrophy (PIA), proposed as the potential precursor to adenocarcinoma due to its proliferative nature, is prevalent in the peripheral zone of the prostate gland, where prostatic intraepithelial neoplasia (PIN) and carcinoma also occur (reviewed in 20, 59, 68, 80, 91). It has been postulated that PIA may transition to carcinoma without any intermediate stage or may lead to carcinoma through PIN. Frequent histological transitions between PIA and PIN have been observed (19, 68). Several crucial prostate tumor suppressor genes such as NKX3.1; CDKN1B, which codes for p27, a cyclin-dependent kinase inhibitor that regulates cell cycle progression; and PTEN (phosphatase and tensin homologue) are all expressed at very low levels in PIA, similar to their expression pattern in PIN and carcinoma (reviewed in 30).

The occurrence of mutations in *p53* and *Rb1* in the early stages of prostate cancer is relatively low (reviewed in 1, 7, 40, 57). This has suggested the possibility that a viral agent like BK virus (BKV), which infects the urinary tract and encodes tumor antigens that inactivate these tumor suppressors, may play a role in the etiology of prostate cancer. This would be very similar to how the E6 and E7 oncogene products of human papillomavirus (HPV) inactivate p53 and pRB, respectively, in cervical carcinomas (13, 22, 27, 37, 50, 74, 75). It has been suggested that

exposure to infectious agents can cause injury to the normal prostate epithelium, leading to the development of PIA (reviewed in 20).

BKV, a member of the polyomavirus family, was first isolated from the urine of a renal transplant patient (28) and infects almost 90% of the human population by early childhood (reviewed in 34, 42, 78). It resides in a subclinical persistent state in the urinary tract of healthy individuals and reactivates in immunosuppressed transplant patients, in whom it is associated with haemorrhagic cystitis and polyomavirus nephropathy (5, 35, 58, 70). BKV transforms rodent cells in culture (65), causes kidney tumors in transgenic mice (15), and immortalizes primary human cells alone (32, 67, 79, 84) or in the presence of other oncogenes such as c-*ras* (60) and adenovirus *E1A* (92). A possible role for BKV in human cancers is controversial because such a high percentage of the human population is exposed to the virus at a very early age, precluding the use of epidemiologic methods to test an association (reviewed in 16)

The genome of BKV is divided into early, late, and regulatory regions and codes for at least six proteins, two from the early region and four from the late region. The early proteins, large tumor antigen (TAg) and small tumor antigen (tAg), are the first to be expressed during infection. When TAg accumulates to high levels, it initiates viral DNA replication in the cell nucleus by recruiting the DNA polymerase α/primase complex to the viral origin of DNA replication, shuts off early gene transcription, and stimulates expression of the late genes, VP1, VP2, VP3, and agnoprotein (reviewed in 36). Under conditions in which viral replication is inhibited, BKV induces oncogenesis through the expression of its two tumor antigens (reviewed in 88). TAg promotes cellular transformation by interfering with the tumor suppressor functions of p53 and pRB (reviewed in 3). TAg upregulates p53 levels in the cell by stabilizing the protein, but functionally inactivates it by sequestering it in an inert form (reviewed in 45, 46, 49, 63, reviewed

in 93). Therefore, expression of TAg and subsequent inactivation of p53 mimic the same phenotypic effect as those caused by mutations in the *p53* gene. Similarly, TAg functionally inactivates pRB by binding it and causing the release of E2F (reviewed in 4). tAg induces tumorigenesis and promotes anchorage-independent growth of transformed cells by the negative regulation of protein phosphatase 2A (64, 95).

In a previous report, utilizing *in situ* analysis, we demonstrated the presence of BKV DNA sequences in epithelial cells of benign and PIA ducts of cancerous prostate specimens (17). Additionally, BKV TAg was expressed specifically in the atrophic epithelial cells but not in the normal epithelium. TAg was detected in the cytoplasm and co-localized with p53 in the atrophic cells, suggesting that neither protein could carry out its normal nuclear function. However, BKV was not detected in the more advanced stages of cancer progression. In the present study we have extended our analysis to non-cancerous prostates. In normal prostates, BKV was present at a lower frequency than in cancerous ones, and TAg expression is only observed in specimens containing PIA and PIN lesions. We also detected TAg expression in the same PIA ducts containing BKV DNA, confirming that the TAg is that of BKV. Utilizing laser capture microdissection on cancerous prostates, we further show that the *p53* gene from TAg-expressing PIA cells was wild type, whereas tumor cells expressing nuclear p53 contained a mix of wild type and mutant *p53* genes. Additionally, we demonstrate that the nuclear localization sequences (NLS) of cytoplasmically localized TAg and p53 were wild type, indicating that the sequestration of TAg and p53 in the cytoplasm was not due to mutations in the NLS of these genes. Together, these results support a causal role for BKV in PIA and the early development of prostate cancer.

MATERIALS AND METHODS

Human Tissue Specimens.

Paraffin-embedded adenocarcinoma prostate resection specimens from radical prostatectomies and cystoprostatectomy specimens from bladder cancer patients with the diagnosis of muscle invasive high grade urothelial carcinoma, with no prostate cancer histology, were obtained from the Tissue Procurement Core at the University of Michigan Comprehensive Cancer Center. One section from each of the specimens was stained with hematoxylin and eosin and was evaluated for the presence of benign, atrophic, or tumor cells by the pathologist. Additionally, autopsy specimens were obtained commercially as a normal prostate tissue microarray from US BIOMAX, Inc.

DNA Extraction and PCR amplification.

DNA extraction was performed using the method previously described by Das *et al.* (2004) in a BKV-free area and cross-contamination was avoided by frequent changing of gloves between samples. The sequences of the oligonucleotide primers used for these studies are listed in Table 1. The BKV early region oligonucleotide probe for ISH (BKV $_{(4434\text{-}4478)}$), and a scrambled control probe with the same length and G+C content were characterized in our previous study (17).

Extracted DNA from entire thin tissue sections was amplified using Titanium^{TM} Taq DNA polymerase (Clontech) in a ThermoHybaid thermocycler (Px2). All reactions were performed in a final volume of 100 μL containing 200 nM each primer, 200 μM dNTPs, 2 μL template and 1U polymerase in 0.5X buffer (20 mM Tricine-KOH, pH 8.0, 8 mM KCl, 1.75 mM $MgCl_2$ and 1.87 μg/mL BSA). For TAg NLS amplification, two rounds of 45 cycles each were used. The template for second round amplification consisted of 30 μL product from the first round amplification. The

program consisted of initial denaturation for 5 mins. at 94^{o}C followed by denaturation at 94^{o}C for 30 secs, annealing at 45^{o}C for 30 secs, and elongation at 72^{o}C for 1 min., followed by final elongation at 72^{o}C for 7 minutes. For p53 gene amplification, 1X buffer was used, with two rounds of 45 cycle amplification. The program for exons 5 through 8 consisted of initial denaturation for 5 mins. at 94^{o}C followed by denaturation at 94^{o}C for 30 secs, annealing at 60^{o}C for 30 secs, and elongation at 72^{o}C for 1 min., followed by final elongation at 72^{o}C for 7 mins. The program for exon 9 was the same except that the annealing temperature was 51^{o}C. The negative control tube contained all the PCR components except DNA template. p53 NLS amplification was done using identical PCR conditions of exons 7 through 8 and 9.

Sequence Analysis.

PCR products were separated by agarose gel electrophoresis, extracted (Qiaquick gel extraction kit, Qiagen), and sequenced by the DNA Sequencing Core at the University of Michigan. Sequences were analyzed using Lasergene software from DNA Star.

Renal Proximal Tubular Epithelial (RPTE) Cells.

RPTE cells were grown and infected with BKV on two well chamber slides (Fisher) as previously reported (47). Cells were fixed and stained 4 days after infection as previously published (17).

***In Situ* DNA Hybridization (ISH) and Immunohistochemistry (IHC).**

ISH and IHC were performed using our previously published protocol (17). For IHC with anti-VP1, 1% SDS retrieval was performed for 8 mins. at room temperature. Monoclonal antibody anti-VP1 (P5G6 BKV9VP1), a gift from Denise Galloway, was used at a 1:600 dilution.

Immuno-Laser Capture Microdissection.

Formalin-fixed, paraffin-embedded 4 μm prostate tissue sections were deparaffinized and rehydrated using our previously published protocol. Immunohistochemistry was performed as previously described except that the antibody dilution for the anti-TAg was 1:300. After IHC and counterstaining with hematoxylin, the sections were dehydrated in graded ethanol solutions (95% ethanol for 2 x 5 mins., 100% ethanol for 3 x 5 mins.) and cleared in xylene for 3 x 5 mins. The slides were air dried for 30 min. in a fume hood and stored in a desiccator until laser capture microdissection. Laser capture was performed using the PIXCELL II Laser Capture Microscope from Arcturus Engineering. The TAg positive PIA cells and nuclear p53 immunolabeled areas were visualized directly, after which the laser pulse was applied to activated thermoplastic film mounted on LCM caps to capture cells of interest. The following parameters were set on the PixCell II LCM system: laser spot size 7.5 μm; power 85 mW; current 250 mV. DNA was extracted from approximately 2500 captured cells by treating with 40 μL of proteinase K buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, 1% Tween 20 and 0.1 mg/mL proteinase K) for 48 hrs at 45^{o}C. Proteinase K was first inactivated at 95^{o}C for 10 mins. The LCM DNA extracts were flash spun in a microcentrifuge and stored at -20^{o}C. A 20 μL aliquot of the LCM DNA template was routinely used for first round PCR amplification.

RESULTS

BKV is present at a significantly higher frequency in cancerous prostates

If BKV plays a role in prostate cancer progression, one would expect to find it at lower frequencies in normal prostates than in cancerous prostates. For these experiments, cancerous prostate is defined as a prostate that has been surgically removed due to a diagnosis of prostate cancer and histologically confirmed to contain malignant glands. Normal prostate is defined as a prostate that has been surgically removed as part of a resection to remove a cancerous bladder (cystoprostatectomy) or during autopsy and histologically confirmed to contain only non-malignant glands.

We initially analyzed the normal prostates using the same *in situ* hybridization (ISH) technique that we previously used with cancerous prostates (17). BKV DNA was absent in the majority of the normal specimens (Figure 1A). However, in a small number of normal specimens, BKV DNA was observed both in normal and atrophic epithelial cells, as demonstrated by nuclear hybridization to the BKV-specific probe (Figure 2, panels B, E and G) but not to the scrambled probe (Figure 2, panels A and D). We did not detect any BKV signal in the stromal cells. The ISH studies on normal prostate demonstrated the presence of BKV DNA sequences in 4/15 of the specimens, which was significantly lower than the frequency of detection in cancerous prostate (Table 2).

Next, we used immunohistochemistry (IHC) with a specific anti-TAg monoclonal antibody to determine whether TAg was being expressed in the normal prostate tissue specimens (Figure 1B-C). In the majority of the normal prostate tissue specimens there was no expression of TAg (panel B). However, in a small number of normal specimens there was expression of TAg in the cytoplasm of atrophic epithelium (panel C). The staining pattern of the atrophic cells was identical

to that observed with cancerous prostate. The IHC analysis of normal prostate demonstrated the expression of TAg in 4/29 of the specimens, which was significantly lower than cancerous prostate (Table 2). We also examined the status of the p53 protein in the normal prostate tissue specimens. In our previous report, we had observed the cytoplasmic localization of p53 in atrophic cells in the cancerous prostate specimens that also expressed TAg. Representative IHC of anti-p53 staining on a serial section from the same normal prostate that was positive for TAg is shown in Figure 1D. p53 expression was cytoplasmic, localized specifically to the atrophic cells that express TAg, and was detectable only in the TAg-positive specimens (Table 3). The ISH and the IHC analysis from normal prostate tissue specimens suggest that BKV is not highly prevalent in the epithelium of normal prostates.

We wanted to confirm whether TAg was expressed in the same ducts that contained BKV DNA. To determine this, we aligned ISH and IHC slides from serial sections that showed the presence of viral DNA and TAg expression (Figure 2). In a BKV-positive specimen, BKV DNA (panels B and G) and TAg expression (panels C and H) were observed in the same PIA duct. In contrast, a normal duct from the same BKV-positive specimen showed robust nuclear staining with the BKV probe (panel E) but lacked TAg expression (panel F).

Wild type *p53* gene in atrophic cells expressing TAg

We next wanted to test the hypothesis that TAg inactivates p53 in the early stages of prostate cancer, specifically PIA. Our hypothesis regarding the status of the *p53* gene in BKV-related prostate cancer is derived from the situation in another virally induced cancer, cervical carcinoma. Like TAg, the HPV E6 oncoprotein inactivates p53. HPV-induced cancers contain wild type p53 while HPV-negative cancers have mutant p53 (13, 27, 37, 74). We predicted that in BKV TAg-expressing cells, a wild type *p53* gene would be observed, and in BKV TAg-

negative tumor cells with detectable nuclear p53, a mutant *p53* gene would be detected. To analyze this, we designed PCR primers to amplify exons 5-9 of the *p53* gene. This spans the sequence-specific DNA binding domain of p53; these exons are the sites for the most frequent mutations in the *p53* gene in various cancers including prostate (reviewed in 21, 94). Utilizing laser capture microdissection (LCM), BKV TAg-positive atrophic cells from PIA and BKV TAg-negative tumor cells expressing nuclear p53 were isolated from cancerous prostate tissue sections immunostained with either anti-TAg or anti-p53, respectively. Amplification was performed both with DNA extracted from entire thin tissue sections and from the laser-captured cells, and the products were sequenced. The entire thin tissue sections contain a mixture of normal, atrophic, tumor, and stromal cells. In both the TAg-positive and TAg-negative captured specimens there was slight stromal cell contamination (Figure 3; C and F). Chromatograms were carefully examined for the presence or absence of mutations in the *p53* gene. A partial comparative chromatogram of exon 5 of the *p53* gene from isolated TAg-positive cells and entire thin tissue section of one specimen shows that the *p53* sequence is wild type in the laser-captured atrophic cells expressing TAg, whereas it is a mixture of wild type and mutant in the entire section (Figure 3). All the laser captured TAg-positive PIA cells we analyzed contained wild type *p53*, whereas cells from entire thin sections of some of these specimens contained a mixture of wild type and mutant *p53* (Table 4). In contrast, captured tumor cells from a specimen that was BKV TAg negative but expressed nuclear p53 contained a mix of wild type and mutant *p53* (specimen #C6). This may be due to heterozygosity at the locus.

Absence of BKV replication marker, VP1 in TAg expressing cancerous prostates

The exclusive cytoplasmic localization of BKV TAg suggested that viral replication, which relies on the host DNA synthetic machinery in the nucleus, cannot be occurring. To test this, IHC analysis was performed on a subset of the TAg-positive specimens using antibody to the viral late protein, VP1, which is a BKV replication marker (Figure 4). We did not detect any positive signal in the tissue specimens with antibody to VP1, indicating that viral replication was not occurring. As a positive control, BKV-infected human renal proximal tubular epithelial cells were analyzed in parallel (panels C-D). Finally, due to the cytoplasmic localization of the TAg and p53, we determined the integrity of the NLS of these two proteins. To accomplish this, DNA was extracted from three TAg-positive tissue specimens, and nested PCR was used to amplify and sequence the TAg NLS. A wild type NLS was observed in all three specimens (data not shown). Additionally, exons 8 and 9 of the *p53* gene, which contain a bipartite NLS, were sequenced from three specimens. In these specimens, the p53 NLS was also wild type (data not shown).

DISCUSSION

The experiments in this study tested the hypothesis that BKV plays a role in the etiology of prostate cancer in its early stages. In our initial *in situ* analysis with cancerous prostates, we had observed the presence of BKV DNA in normal and PIA epithelium, with TAg expression specifically localized to the atrophic but not the normal epithelium (17). BKV was not detected and was assumed to be lost as the cancer advanced to PIN or invasive carcinoma. We have extended our previous studies to determine if BKV is also present in non-diseased prostates, or whether its detection in cancerous tissue is simply a reflection of its ubiquitous presence in the human population. When we compared the presence of BKV between diseased and non-diseased prostates, BKV was found at a higher frequency in cancerous prostates. Viral DNA was detected in 79% of cancerous prostates but only in 27% of non-diseased prostates, and TAg expression was observed in 47% of cancerous prostates but only in 14% of non-diseased prostates. Fisher's exact test both for ISH (p=0.007) and IHC (p= 0.008) shows a significant difference between the presence and expression of BKV in normal and cancerous prostates. Interestingly, in both types of specimen, TAg expression was only detected in PIA cells. In the non-diseased prostates, however, TAg expression in PIA lesions was only observed in those specimens also containing PIN lesions. Additionally, TAg expression is localized to the cytoplasm in both types of specimen, but the NLS is wild type. The comparative analysis between cancerous and non-diseased prostate supports our hypothesis that BKV is a cofactor in PIA.

PIA has been observed in the vicinity of carcinoma lesions and has been reported to sometimes merge with adenocarcinoma (18, reviewed in 20). Epithelial cells in PIA are highly proliferative, exhibit phenotypic characteristics that are intermediate between secretory and basal cells, and have been proposed to be precursors to prostatic neoplastic transformation (91). Merging

of focal areas of PIA with PIN has also been reported (18). Interestingly, occurrence of mutations in *p53* or *Rb1* in PIA is low, which supports a viral cause for the transition of benign epithelium to PIA lesions through the inactivation of these tumor suppressors by viral oncoproteins.

There are two additional reports on the presence of BKV DNA in prostate carcinomas (44, 96). Zambrano *et al*. (2002) demonstrated the presence of BKV DNA in 3/12 prostate specimens by utilizing PCR, and Lau *et al.* (2007) detected BKV DNA in tumor cells in 2/30 prostate specimens using ISH. Our previous study, however, was the first to demonstrate the presence of both viral DNA and oncoprotein expression in PIA lesions of neoplastic prostate (17). In the current report, we specifically examined expression of TAg in the same PIA ducts that contain BKV DNA, further supporting our previous conclusion that the TAg is indeed that of BKV. Additionally, in this study, we show that although the normal ducts may have BKV DNA, there is no expression of TAg in those ducts. This suggests the intriguing possibility that BKV infects the normal epithelium and resides in a latent state, and that activation of TAg expression in PIA may promote the transition from benign to atrophic, ultimately leading to prostate cancer. The fact that TAg is only detected in the PIA of non-diseased prostates that also have signs of PIN also supports a possible link between BKV and cancerous lesions. It is tempting to speculate here that these non-diseased prostates which have PIN are already on their way to cancer, and TAg expression may act as a co-factor that promotes this transition. Interestingly, prostate cancer is a slowly progressing disease, focal areas of atrophy are a common occurrence of the ageing prostate (reviewed in 20, 26, 29, 48, 71), and BKV is a relatively poor transforming agent (6, 33).

Detection of p53 by IHC usually requires that the p53 be stabilized in some manner (23, 24). Two known means of stabilization are binding by TAg (reviewed in 63) and mutation (reviewed in 61, reviewed in 82). Our analysis of the *p53* gene from laser captured TAg-

positive PIA cells and TAg-negative tumor cells with nuclear p53 is therefore relevant to whether the virus is involved in oncogenesis. The *p53* sequence is wild type in laser-captured PIA cells expressing TAg. This is similar to the status of *p53* genes in cervical carcinomas expressing the HPV E6 oncoprotein (reviewed in 86), and suggests that the wild type p53 is being inactivated by sequestration in the cytoplasm. However, when the *p53* sequence was analyzed from entire thin sections from cancerous prostates, a mixture of wild type and mutant *p53* was sometimes observed. We predicted that the mutant *p53* sequence that we observed from TAg-positive thin sections was derived from tumor cells. LCM of p53-positive tumor cells supported this, because we detected a mix of wild type and mutant *p53* in those specimens. In spite of slight stromal cell contamination in the LCM samples, the mutant form of p53 was readily detectable in the specimen expressing nuclear p53. An earlier study using laser capture microdissection demonstrated a very low occurrence of *p53* gene mutations in PIA lesions (90). Since the *p53* gene in TAg-positive cells in cancerous prostates is wild type, we did not perform this analysis in normal specimens. p53 expression in both types of specimen was only detectable in PIA cells and was cytoplasmic despite the protein having a wild type NLS. For p53 to function as a tumor suppressor, its translocation and retention in the nucleus is required (41, 53, 73). Nuclear p53 over-expression was not observed in any of the non-diseased prostates.

It is interesting that TAg, which is normally a nuclear protein, is localized in the cytoplasm of the epithelial cells in these PIA lesions. The tumorigenic potential of an SV40 TAg containing a mutation in the NLS, causing it to remain in the cytoplasm, has been previously reported (reviewed in 10, 43, 62). This cytoplasmic SV40 TAg has the ability to induce tumors in transgenic mice at a rate equivalent to nuclear TAg (62). However, in our studies the NLS of BKV TAg was wild type.

In addition, BKV replication, as measured by VP1 expression, was not observed in the TAg-positive specimens, consistent with the observation of TAg expression in the cytoplasm.

Additionally, cytoplasmic p53 expression in transformed cells in the presence of a cytoplasmically localized SV40 TAg has been previously reported (43). Cytoplasmic localization of p53 has been also observed in various human tumors (8, 9, 52, 54, 77, 81). This suggests that in certain cancers, functional p53 inactivation occurs by the sequestration of the protein in the cytoplasm, leading to acceleration of tumor progression by the accumulation of chromosomal mutations during cell proliferation.

The fact that the NLS of TAg and p53 were wild type in BKV positive prostates suggests that there is a cellular factor(s) in the cytoplasm that is sequestering both proteins. Wild type p53 has been shown to be retained in the cytoplasm as a result of interactions with proteins like Hdm-2 and heat shock proteins (83). Phosphorylation of serine residues adjacent to the NLS of SV40 TAg by casein kinase II facilitates the nuclear translocation of TAg and this process of nuclear import is inhibited by the $p34^{cdc2}$- mediated phosphorylation of a nearby threonine residue (38, 72, 76). Complexes of $p34^{cdc2}$ and p53 have been observed in TAg-transformed cells (51) and TAg has been shown to stimulate the expression of the *cdc2* gene (12, 56). Interestingly, serine 315 of p53 is adjacent to one of its NLS and is also phosphorylated by $p34^{cdc2}$ (2), and exclusion of p53 from the nucleus due to the phosphorylation of serine-315 was recently demonstrated (69). It is tempting to speculate that in the prostate specimens that express TAg, $p34^{cdc2}$ phosphorylates TAg and/or p53, which impairs the ability of both proteins to translocate to the nucleus.

Based on our findings, we present the following model (Figure 5). BKV infects normal epithelial cells and induces a change of the normal cells to PIA through the expression of TAg; alternatively, the transition to PIA induces TAg expression. This results in induction of

proliferation and sequestration of p53 in the cytoplasm. As the cells proliferate, they accumulate mutations at a higher-than-normal rate due to the absence of p53 activity. Eventually, a cell accumulates enough mutations to completely lose growth control and clonally expands into a tumor. The loss of BKV in the tumor cells could be due to selection against TAg by the immune system, dilution of viral episomes due to lack of replication, or pro-apoptotic effects mediated by TAg that are not compatible with the other growth control mutations in the tumor cells (85, 87, 89), resulting in selection against TAg expression. A similar loss of TAg expression has been reported in studies of TRAMP (TRansgenic Adenocarcinoma of Mouse Prostate) mice, which develop tumors due to expression of SV40 TAg (31). When tumor cells are removed from these animals and grown in culture, TAg expression is lost (25). A similar loss of oncoprotein expression is seen in cancers of the alimentary canal in cattle caused by bovine papillomavirus type 4 (BPV-4): the virus is required to induce papillomas but its presence is not necessary for progression or maintenance of the transformed state (11). Additional work will be required to determine if a causal connection between BKV and prostate cancer exists. If so, the unique viral properties of BKV can be explored for the possibility of prophylactic or therapeutic vaccination, or treatment by designing drugs that target TAg.

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REFERENCES

1. **Abate-Shen, C., and M. M. Shen.** 2000. Molecular genetics of prostate cancer. Genes Dev. **14:**2410-2434.
2. **Addison, C., J. R. Jenkins, and H. W. Stürzbecher.** 1990. The p53 nuclear localisation signal is structurally linked to a p34cdc2 kinase motif. Oncogene **5:**423-426.
3. **Ahuja, D., M. T. Saenz-Robles, and J. M. Pipas.** 2005. SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation. Oncogene **24:**7729-7745.
4. **Ali, S. H., and J. A. DeCaprio.** 2001. Cellular transformation by SV40 large T antigen: interaction with host proteins. Semin Cancer Biol. **11:**15-23.
5. **Barbanti-Brodano, G., F. Martini, M. De Mattei, L. Lazzarin, A. Corallini, and M. Tognon.** 1998. BK and JC human polyomaviruses and simian virus 40: natural history of infection in humans, experimental oncogenicity, and association with human tumors. Adv Virus Res **50:**69-99.
6. **Bollag, B., W. F. Chuke, and R. J. Frisque.** 1989. Hybrid genomes of the polyomaviruses JC virus, BK virus, and simian virus 40: Identification of sequences important for efficient transformation. J Virol **63:**863-872.
7. **Bookstein, R., D. MacGrogan, S. G. Hilsenbeck, F. Sharkey, and D. C. Allred.** 1993. p53 Is Mutated in a Subset of Advanced-Stage Prostate Cancers. Cancer Res **53:**3369-3373.
8. **Bosari, S., G. Viale, P. Bossi, M. Maggioni, G. Coggi, J. J. Murray, and A. K. C. Lee.** 1994. Cytoplasmic Accumulation of p53 Protein: an Independent Prognostic Indicator in Colorectal Adenocarcinomas. J. Natl. Cancer Inst. **86:**681-687.

9. **Bosari, S., G. Viale, M. Roncalli, D. Graziani, G. Borsani, A. K. Lee, and G. Coggi.** 1995. p53 gene mutations, p53 protein accumulation and compartmentalization in colorectal adenocarcinoma. Am J Pathol. **147:**790-798.

10. **Butel, J. S., and D. L. Jarvis.** 1986. The plasma-membrane-associated form of SV40 large tumor antigen: biochemical and biological properties. Biochim Biophys Acta. **865:**171-95.

11. **Campo, M. S., M. H. Moar, M. L. Sartirana, I. M. Kennedy, and W. F. Jarrett.** 1985. The presence of bovine papillomavirus type 4 DNA is not required for the progression to, or the maintenance of, the malignant state in cancers of the alimentary canal in cattle. EMBO J **4:**1819-25.

12. **Chen, H., J. Campisi, and R. Padmanabhan.** 1996. SV40 Large T Antigen Transactivates the Human cdc2 Promoter by Inducing a CCAAT Box Binding Factor. J. Biol. Chem. **271:**13959-13967.

13. **Choo, K. B., and K. Y. Chong.** 1993. Absence of mutation in the p53 and the retinoblastoma susceptibility genes in primary cervical carcinoma. Virology **193:**1042-1046.

14. **Chrisofos, M., A. G. Papatsoris, A. Lazaris, and C. Deliveliotis.** 2007. Precursor lesions of prostate cancer. Crit Rev Clin Lab Sci. **44:**243-270.

15. **Dalrymple, S. A., and K. L. Beemon.** 1990. BK virus T antigens induce kidney carcinomas and thymoproliferative disorders in transgenic mice. J Virol. **64:**1182-1191.

16. **Das, D., and M. J. Imperiale.** 2006. BK virus and human tumors, p. 1-26. *In* M. Tognon (ed.), Viral Oncogenesis. Research Signpost, Trivandrum.

17. **Das, D., R. B. Shah, and M. J. Imperiale.** 2004. Detection and expression of human BK virus sequences in neoplastic prostate tissues. Oncogene **23:**7031-7046.

18. **De Marzo, A. M., T. L. DeWeese, E. A. Platz, A. K. Meeker, M. Nakayama, J. I. Epstein, W. B. Isaacs, and W. G. Nelson.** 2004. Pathological and molecular mechanisms of prostate carcinogenesis: Implications for diagnosis, detection, prevention, and treatment. Journal Of Cellular Biochemistry **91:**459-477.

19. **De Marzo, A. M., V. L. Marchi, J. I. Epstein, and W. G. Nelson.** 1999. Proliferative inflammatory atrophy of the prostate - Implications for prostatic carcinogenesis. American Journal Of Pathology **155:**1985-1992.

20. **De Marzo, A. M., E. A. Platz, S. Sutcliffe, J. Xu, H. Gronberg, C. G. Drake, Y. Nakai, W. B. Isaacs, and W. G. Nelson.** 2007. Inflammation in prostate carcinogenesis. Nat Rev Cancer **7:**256-269.

21. **Dong, J. T.** 2006. Prevalent mutations in prostate cancer. Journal of Cellular Biochemistry **97:**433-447.

22. **Dyson, N., P. M. Howley, K. Münger, and E. Harlow.** 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243:**934-937.

23. **Esrig, D., D. Elmajian, S. Groshen, J. A. Freeman, J. P. Stein, S.-C. Chen, P. W. Nichols, D. G. Skinner, P. A. Jones, and R. J. Cote.** 1994. Accumulation of Nuclear p53 and Tumor Progression in Bladder Cancer. N Engl J Med **331:**1259-1264.

24. **Esrig, D., C. H. d. Spruck, P. W. Nichols, B. Chaiwun, K. Steven, S. Groshen, S. C. Chen, D. G. Skinner, P. A. Jones, and R. J. Cote.** 1993. p53 nuclear protein accumulation correlates with mutations in the p53 gene, tumor grade, and stage in bladder cancer. Am J Pathol **143:**1389-1397.

25. **Foster, B. A., J. R. Gingrich, E. D. Kwon, C. Madias, and N. M. Greenberg.** 1997. Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Cancer Res. **57:**3325-30.

26. **Franks, L. M.** 1954. Atrophy And Hyperplasia In The Prostate Proper. Journal Of Pathology And Bacteriology **68:**617-621.

27. **Fujita, M., M. Inoue, O. Tanizawa, S. Iwamoto, and T. Enomoto.** 1992. Alteration of the p53 Gene in Human Primary Cervical Carcinoma with and without Human Papillomavirus Infection. Cancer Res **52:**5323-5328.

28. **Gardner, S. D., A. M. Field, D. V. Coleman, and B. Hulme.** 1971. New human papovavirus (B.K.) isolated from urine after renal transplantation. Lancet **1:**1253-7.

29. **Gardner, W. A., Jr, and D. E. Culberson.** 1987. Atrophy and proliferation in the young adult prostate. J. Urol. **137:**53-56.

30. **Gonzalgo, M. L., and W. B. Isaacs.** 2003. Molecular pathways to prostate cancer. Journal Of Urology **170:**2444-2452.

31. **Greenberg, N. M., F. DeMayo, M. J. Finegold, D. Medina, W. D. Tilley, J. O. Aspinall, G. R. Cunha, A. A. Donjacour, R. J. Matusik, and J. M. Rosen.** 1995. Prostate cancer in a transgenic mouse. Proc Natl Acad Sci U S A. **92:**3439-3443.

32. **Grossi, M. P., A. Caputo, G. Meneguzzi, A. Corallini, L. Carra, M. Portolani, M. Borgatti, G. Milanesi, and G. Barbanti-Brodano.** 1982b. Transformation of human embryonic fibroblasts by BK virus, BK virus DNA, and a subgenomic BK virus DNA fragment. J Gen Virol **63:**393-403.

33. **Harris, K. F., J. B. Christensen, and M. J. Imperiale.** 1996. BK virus large T antigen: Interactions with the retinoblastoma family of tumor suppressor proteins and effects on cellular growth control. J Virol **70:**2378-2386.

34. **Hirsch, H. H.** 2005. BK Virus: Opportunity Makes a Pathogen. Clin Infect Dis **41:**354-360.

35. **Hirsch, H. H.** 2002. Polyomavirus BK nephropathy: a (re-)emerging complication in renal transplantation. Am J Transplant. **2:**25-30.

36. **Imperiale, M. J., and E. O. Major.** 2007. Polyomaviruses, p. 2263-2298. *In* D. M. Knipe and P. M. Howley (ed.), Fields Virology, Fifth ed, vol. 2. Lippincott Williams & Wilkins, Philadelphia.

37. **Iwasaka, T., M. Oh-uchida, N. Matsuo, M. Yokoyama, K. Fukuda, K. Hara, K. Fukuyama, K. Hori, and H. Sugimori.** 1993. Correlation between HPV positivity and state of the p53 gene in cervical carcinoma cell lines. Gynecol. Oncol. **48:**104-109.

38. **Jans, D., M. Ackermann, J. Bischoff, D. Beach, and R. Peters.** 1991. p34cdc2-mediated phosphorylation at T124 inhibits nuclear import of SV-40 T antigen proteins. J Cell Biol. **115:**1203-1212.

39. **Jemal, A., R. Siegel, E. Ward, T. Murray, J. Xu, and M. J. Thun.** 2007. Cancer Statistics, 2007. CA Cancer J Clin **57:**43-66.

40. **Jin-Tang, D.** 2006. Prevalent mutations in prostate cancer. Journal of Cellular Biochemistry **97:**433-447.

41. **Knippschild, U., M. Oren, and W. Deppert.** 1996. Abrogation of wild-type p53 mediated growth-inhibition by nuclear exclusion. Oncogene **12:**1755-1765.

42. **Knowles, W. A., P. Pipkin, N. Andrews, A. Vyse, P. Minor, D. W. Brown, and E. Miller.** 2003. Population-based study of antibody to the human polyomaviruses BKV and JCV and the simian polyomavirus SV40. J Med Virol. **71:**115-123.

43. **Lanford, R., C. Wong, and J. Butel.** 1985. Differential ability of a T-antigen transport-defective mutant of simian virus 40 to transform primary and established rodent cells. Mol Cell Biol. **5:**1043-50.

44. **Lau, S. K., S. F. Lacey, Y.-Y. Chen, W.-G. Chen, and L. M. Weiss.** 2007. Low frequency of BK virus in prostatic adenocarcinomas. APMIS **115:**743-749.

45. **Lin, J. Y., and D. T. Simmons.** 1990. Transformation by simian virus 40 does not involve the mutational activation of p53 to an oncogenic form. Virology. **176:**302-305.

46. **Liu, X., and R. Marmorstein.** 2006. When viral oncoprotein meets tumor suppressor: a structural view. Genes Dev. **20:**2332-2337.

47. **Low, J. A., B. Magnuson, B. Tsai, and M. J. Imperiale.** 2006. Identification of Gangliosides GD1b and GT1b as Receptors for BK Virus. J. Virol. **80:**1361-1366.

48. **McNeal, J. E.** 1988. Normal histology of the prostate. Am J Surg Pathol. **12:**619-633.

49. **Mietz, J. A., T. Unger, J. M. Huibregtse, and P. M. Howley.** 1992. The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. EMBO J. **11:**5013-5020.

50. **Milde-Langosch, K., K. Albrecht, S. Joram, H. Schlechte, M. Giessing, and T. Löning.** 1995. Presence and persistence of HPV infection and p53 mutation in cancer of the cervix uteri and the vulva. Int J Cancer **63:**639-645.

51. **Milner, J., A. Cook, and J. Mason.** 1990. p53 is associated with p34cdc2 in transformed cells. EMBO J. **9:**2885-2889.

52. **Moll, U. M., M. LaQuaglia, J. Bénard, and G. Riou.** 1995. Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. Proc Natl Acad Sci U S A. **92:**4407-4411.

53. **Moll, U. M., A. G. Ostermeyer, R. Haladay, B. Winkfield, M. Frazier, and G. Zambetti.** 1996. Cytoplasmic sequestration of wild-type p53 protein impairs the G1 checkpoint after DNA damage. Mol Cell Biol. **16:**1126-1137.

54. **Moll, U. M., G. Riou, and A. J. Levine.** 1992. Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. Proc Natl Acad Sci U S A. **89:**7262-7266.

55. **Montironi, R., R. Mazzucchelli, A. Lopez-Beltran, L. Cheng, and M. Scarpelli.** 2007. Mechanisms of disease: high-grade prostatic intraepithelial neoplasia and other proposed preneoplastic lesions in the prostate. Nat Clin Pract Urol. **4:**321-332.

56. **Oshima, J., K. Steinmann, J. Campisi, and R. Schlegel.** 1993. Modulation of cell growth, p34cdc2 and cyclin A levels by SV-40 large T antigen. Oncogene **8:**2987-2993.

57. **Osman, I., M. Drobnjak, M. Fazzari, J. Ferrara, H. I. Scher, and C. Cordon-Cardo.** 1999. Inactivation of the p53 Pathway in Prostate Cancer: Impact on Tumor Progression. Clin Cancer Res **5:**2082-2088.

58. **Padgett, B. L., and D. L. Walker.** 1976. New human papovaviruses. Prog Med Virol. **22:**1-35.

59. **Palapattu, G. S., S. Sutcliffe, P. J. Bastian, E. A. Platz, A. M. De Marzo, W. B. Isaacs, and W. G. Nelson.** 2005. Prostate carcinogenesis and inflammation: emerging insights. Carcinogenesis **26:**1170-1181.

60. **Pater, A., and M. M. Pater.** 1986. Transformation of primary human embryonic kidney cells to anchorage independence by a combination of BK virus DNA and the Harvey-ras oncogene. J Virol. **58:**680-3.

61. **Petitjean, A., M. I. W. Achatz, A. L. Borresen-Dale, P. Hainaut, and M. Olivier.** 2007. TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. Oncogene **26:**2157-2165.

62. **Pinkert, C. A., R. L. Brinster, R. D. Palmiter, Wong C, and J. S. Butel.** 1987. Tumorigenesis in transgenic mice by a nuclear transport-defective SV40 large T-antigen gene. Virology **160:**169-75.

63. **Pipas, J. M., and A. J. Levine.** 2001. Role of T antigen interactions with p53 in tumorigenesis. Semin Cancer Biol. **11:**23-30.

64. **Porras, A., J. Bennett, A. Howe, K. Tokos, N. Bouck, B. Henglein, S. Sathyamangalam, B. Thimmapaya, and K. Rundell.** 1996. A novel simian virus 40 early-region domain mediates transactivation of the cyclin A promoter by small-t antigen and is required for transformation in small-t antigen-dependent assays. J. Virol. **70:**6902-6908.

65. **Portolani, M., and M. Borgatti.** 1978. Stable transformation of mouse, rabbit and monkey cells and abortive transformation of human cells by BK virus, a human papovavirus. J Gen Virol. **38:**369-74.

66. **Preacher, K. J., and N. E. Briggs.** 2001. Calculation for Fisher's Exact Test: An interactive calculation tool for Fisher's exact probability test for 2 x 2 tables [Computer software]. Available from http:// www.quantpsy.org

67. **Purchio, A. F., and G. C. Fareed.** 1979. Transformation of human embryonic kidney cells by human papovarirus BK. J Virol. **29:**763-9.

68. **Putzi, M. J., and A. M. De Marzo.** 2000. Morphologic transitions between proliferative inflammatory atrophy and high-grade prostatic intraepithelial neoplasia. Urology **56:**828-832.

69. **Qu, L., S. Huang, D. Baltzis, A.-M. Rivas-Estilla, O. Pluquet, M. Hatzoglou, C. Koumenis, Y. Taya, A. Yoshimura, and A. E. Koromilas.** 2004. Endoplasmic reticulum stress induces p53 cytoplasmic localization and prevents p53-dependent apoptosis by a pathway involving glycogen synthase kinase-3{beta}. Genes Dev. **18:**261-277.

70. **Reese, J. M., M. Reissig, R. W. Daniel, and K. V. Shah.** 1975. Occurrence of BK Virus and BK Virus-Specific Antibodies in the Urine of Patients Receiving Chemotherapy for Malignancy. Infect Immun. **11:**1375-1381.

71. **Rich, A. R.** 1934. On the frequency of occurrence of occult carcinoma of the prostate. J. Urol. **33:**215-223.

72. **Rihs, H., D. Jans, H. Fan, and R. Peters.** 1991. The rate of nuclear cytoplasmic protein transport is determined by the casein kinase II site flanking the nuclear localization sequence of the SV40 T-antigen. EMBO J. **10:**633-639.

73. **Ryan, J. J., E. Prochownik, C. A. Gottlieb, I. J. Apel, R. Merino, G. Nunez, and M. F. Clarke.** 1994. c-myc and bcl-2 Modulate p53 Function by Altering p53 Subcellular Trafficking During the Cell Cycle. PNAS **91:**5878-5882.

74. **Scheffner, M., K. Munger, J. C. Byrne, and P. M. Howley.** 1991. The state of the p53 and retinoblastoma genes in cervical carcinoma cell lines. Proc. Natl. Acad. Sci. USA. **88:**5523-5527.

75. **Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell **63:**1129-1136.

76. **Scheidtmann, K., J. Schickedanz, G. Walter, R. Lanford, and J. Butel.** 1984. Differential phosphorylation of cytoplasmic and nuclear variants of simian virus 40 large T antigen encoded by simian virus 40-adenovirus 7 hybrid viruses. J Virol **50:**636-640.

77. **Schlamp, C. L., G. L. Poulsen, T. M. Nork, and R. W. Nickells.** 1997. Nuclear exclusion of wild-type p53 in immortalized human retinoblastoma cells. J. Natl. Cancer Inst. **89:**1530-1536.

78. **Shah, K. V., R. W. Daniel, and R. M. Warszawski.** 1973. High prevalence of antibodies to BK virus, an SV40-related papovavirus, in residents of Maryland. J Infect Dis. **128:**784-787.

79. **Shah, K. V., C. Hudson, J. Valis, and J. D. Strandberg.** 1976. Experimental infection of human foreskin cultures with BK virus, a human papovavirus. Proc Soc Exp Biol Med **153:**180-6.

80. **Shah, R., N. R. Mucci, A. Amin, J. A. Macoska, and M. A. Rubin.** 2001. Postatrophic hyperplasia of the prostate gland: neoplastic precursor or innocent bystander? Am J Pathol. **158:**1767-1773.

81. **Soong, R., P. D. Robbins, B. R. Dix, F. Grieu, B. Lim, S. Knowles, K. E. Williams, G. R. Turbett, A. K. House, and B. J. Iacopetta.** 1996. Concordance between p53 protein overexpression and gene mutation in a large series of common human carcinomas. Hum Pathol. **27:**1050-1055.

82. **Soussi, T.** 2007. p53 alterations in human cancer: more questions than answers. Oncogene **26:**2145-2156.

83. **Sun, X. F., H. Zhang, J. Carstensen, A. Jansson, and B. Nordenskjöld.** 1997. Heat shock protein 72/73 in relation to cytoplasmic p53 expression and prognosis in colorectal adenocarcinomas. International Journal of Cancer **74:**600-604.

84. **Takemoto, K. K., H. Linke, T. Miyamura, and G. C. Fareed.** 1979. Persistent BK papovavirus infection of transformed human fetal brain cells. I. Episomal viral DNA in cloned lines deficient in T-antigen expression. J Virol. **29:**1177-85.

85. **Theile, M., and G. Grabowski.** 1990. Mutagenic activity of BKV and JCV in human and other mammalian cells. Arch Virol. **113:**221-233.

86. **Thomas, M., D. Pim, and L. Banks.** 1999. The role of the E6-p53 interaction in the molecular pathogenesis of HPV. Oncogene **18:**7690-7700.

87. **Tognon, M., R. Casalone, F. Martini, M. De Mattei, P. Granata, E. Minelli, C. Arcuri, P. Collini, and V. Bocchini.** 1996. Large T antigen coding sequences of two DNA tumor viruses, BK and SV40, and non random chromosome changes in two glioblastoma cell lines. Cancer Genet Cytogenet. **90:**17-23.

88. **Tognon, M., A. Corallini, F. Martini, M. Negrini, and G. Barbanti-Brodano.** 2003. Oncogene transformation by BK virus and association with human tumors. Oncogene **22:**5192-5200.

89. **Trabanelli, C., A. Corallini, R. Gruppioni, A. Sensi, A. Bonfatti, D. Campioni, M. Merlin, N. Calza, L. Possati, and G. Barbanti-Brodano.** 1998. Chromosomal aberrations induced by BK virus T antigen in human fibroblasts. Virology. **243:**492-6.

90. **Tsujimoto, Y., H. Takayama, N. Nonomura, A. Okuyama, and K. Aozasa.** 2002. Postatrophic hyperplasia of the prostate in Japan: Histologic and immunohistochemical features and *p53* gene mutation analysis. The Prostate **52:**279-287.

91. **van Leenders, G., W. R. Gage, J. L. Hicks, B. van Balken, T. W. Aalders, J. A. Schalken, and A. De Marzo.** 2003. Intermediate cells in human prostate epithelium are enriched in proliferative inflammatory atrophy. American Journal Of Pathology **162:**1529-1537.

92. **Vasavada, R., K. B. Eager, G. Barbanti-Brodano, A. Caputo, and R. P. Ricciardi.** 1986. Adenovirus type 12 early region 1A proteins repress class I HLA expression in transformed human cells. Proc Natl Acad Sci U S A **83:**5257-61.

93. **Vousden, K. H., and C. Prives.** 2005. P53 and prognosis: New insights and further complexity. Cell **120:**7-10.

94. **Weisz, L., M. Oren, and V. Rotter.** 2007. Transcription regulation by mutant p53. Oncogene **26:**2202-2211.

95. **Yu, J., A. Boyapati, and K. Rundell.** 2001. Critical role for SV40 small-t antigen in human cell transformation. Virology **290:**192-198.

96. **Zambrano, A., M. Kalantari, A. Simoneau, J. L. Jensen, and L. P. Villarreal.** 2002. Detection of human polyomaviruses and papillomaviruses in prostatic tissue reveals the prostate as a habitat for multiple viral infections. The Prostate **53:**263-276.

TABLE 1. Primers

Target Gene	Primer	Round	Sequence (5’ to 3’)
p53 exons			
5-6	(13040-13058)	1	TTCCTCTTCCTACAGTACT
	(13493-13475)		GGAGGGCCACTGACAACCA
5-6	(13057-13077)	2	CTCCCCTGCCCTCAACAAGAT
	(13432-13412)		CTCAGGCGGCTCATAGGGCAC
7-8	(13987-14006)	1	TGTTATCTCCTAGGTTGGCT
	(14703-14684)		GGAGCTGGTGTTGTTGGGCA
7-8	(14003-14022)	2	GGCTCTGACTGTACCACCAT
	(14602-14583)		TCCTGCTTGCTTACCTCGCT
7	(14217-14198)	2	ATGGGTAGTAGTATGGAAGA
	(14003-14022)		GGCTCTGACTGTACCACCAT
8	(14591-14572)	2	TACCTCGCTTAGTGCTCCCT
	(14438-14457)		CCTATCCTGAGTAGTGGTAA
9	(14521-14540)	**1**	AGAGGAAGAGAATCTCCGCA
	(14830-14811)		CAGTCAAGAAGAAAACGGCA
	(14796-14777)	2	CTGGAAACTTTCCACTTGAT
	(14579-14598)		ACTAAGCGAGGTAAGCAAGC
BKV TAg NLS	(4670-4649)	1	ATTGGAGAAACTCCCTTCAGAG
	(4315-4334)		AGTATACACAGCAAAGCAGG
	(4467-4443)	2	GATGAAGAAGCAACAGCAGATTCTC
	(4358-4382)		GCTTGACTAAGAAACTGGTGTAGAT

NLS, Nuclear Localization Sequence

TABLE 2. Comparison of cancerous and normal prostate specimens

	Normal	Cancer	*p*-value
BKV DNA (pos)	4	11	
BKV DNA (neg)	11	3	
fraction*	0.27	0.79	0.007
TAg (pos)	4	13	
TAg (neg)	25	15	
fraction^{+}	0.14	0.46	0.008

p-value was obtained by using Fisher's exact probability test (66)
*fraction of BKV positive specimens
$^{+}$fraction of TAg positive specimens

TABLE 3. Summary of normal prostate data

Specimen	Histology	ISH	TAg	p53
1	N	Pos	Neg	Neg
2	N	ND	Neg	Neg
3	A	ND	Neg	Neg
4	N, A	ND	Neg	Neg
5	N, A	ND	Neg	Neg
6	N, A	ND	Neg	Neg
7	N, A	Neg	Neg	Neg
8	N, A	Pos	Neg	Neg
9	N, A	ND	Neg	Neg
10	N, A	Neg	Neg	Neg
11	N, A	ND	Neg	Neg
12	N, A	ND	Neg	Neg
13	N, A, P	ND	Neg	Neg
14	N, A, P	Neg	Neg	Neg
15	N, A, P	Pos	Pos (A)	Pos (A)
16	N, A, B, P	Pos	Pos (A)	Pos (A)
17	N, A, B, P	ND	Pos (A)	Pos (A)
18	N, A, B, P	ND	Pos (A)	Pos (A)
19	N, A, B, P	ND	Neg	Neg
20	N, A, B, P	Neg	Neg	Neg
21	N, A, B, P	Neg	Neg	Neg
22	N, A, B, P	ND	Neg	Neg
23	N, T	ND	Neg	Neg

N, normal; A, (PA, prostatic atrophy and PIA, proliferative inflammatory atrophy); B, benign prostatic hyperplasia; P, PIN, prostatic intraepithelial neoplasia; ND, not determined; ISH, *in situ* hybridization with a early region DNA probe for BKV; T, in this specimen section there was a very small area of incidental tumor

TABLE 4. *p53* Sequence Analysis

Specimen	TAg	p53	Exon	Entire thin section	LCM
C1	Cyto	Cyto	5-6	wt, mt (Thr 140 to Ile)	wt
			9	wt	ND
C2	Cyto	Cyto	5-6	wt	wt
			7-8	wt	wt
			9	wt	wt
C3	Cyto	Cyto	5-6	wt	wt
			7-8	wt	wt*
			9	wt	wt
C4	Cyto	Cyto	5-6	mt (Pro 191 to Leu)	wt
			7-8	wt	wt
			9	wt	wt
C5	Cyto	Cyto	5-6	ND	wt
			7-8	wt	wt*
			9	wt	wt
C6		Nuc	5-6	wt	wt
			7-8	wt	wt
			9	wt	wt, mt (Pro 318 to Ser)
C7		Nuc	5-6	wt	wt
			7-8	wt	wt*
			9	wt	wt
C8		Nuc	5-6	wt	wt
			7-8	wt	wt*

wt, wild type; mt, mutant; Nuc, Nuclear localization; Cyto, Cytoplasmic localization; ND, Not Determined; *exon 8 sequence of these specimens could be amplified but good quality sequence could not be obtained; Specimen, cancerous prostate; (C1-C5), LCM of TAg+ PIA cells; (C6-C8), LCM of TAg- tumor cells

FIGURE LEGENDS

Figure 1. *In situ* hybridization and immunohistochemistry of normal prostates. (**A**), section stained with BKV-specific probe. (**B**), section immunostained with anti-TAg antibody. (**C**), PIA duct from a section that was positive for the presence of BKV DNA, showing immunostaining with anti-TAg antibody. (**D**), PIA duct from the same specimen as (**C**), immunostained with anti-p53 antibody. Magnification: x 400 (**A**, **C** and **D**); x 100 (**B**)

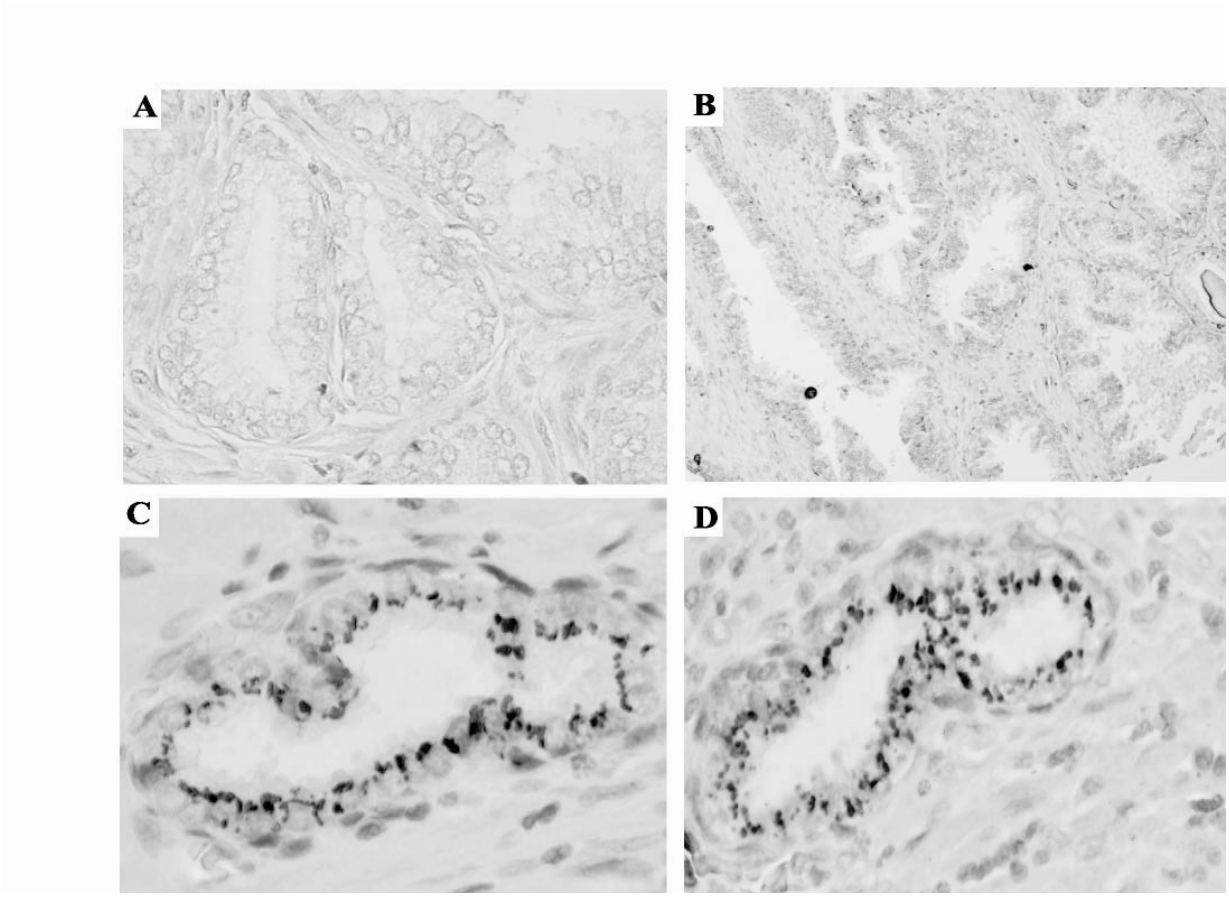
Figure 2. Alignment of ducts for the presence of BKV DNA and TAg expression. (**A-C**) are serial sections of the same atrophic duct showing hybridization with scrambled probe (**A**), BKV-specific probe (**B**) and immunostaining with anti-TAg antibody (**C**). (**D-F**) are serial sections of the same normal duct from the same specimen showing hybridization with scrambled probe (**D**), BKV-specific probe (**E**) and immunostaining with anti-TAg (**F**). (**G**) and (**H**) are identical PIA ducts of the same specimen showing hybridization with BKV-specific probe and immunostaining with anti-TAg, respectively. Magnification: x 100 (**A-C**); x 200 (**D-H**). Sections **A**, **B**, **D**, **E** and **F** were counterstained with contrast red and **C**, **F** and **H** were counterstained with hematoxylin. The green stain in **D**, **E** and **F** is orientation ink used by pathologist to maintain the orientation of the exterior surface of the prostate in a paraffin-embedded block. Positive staining for ISH is dark red/black and for IHC is brown.

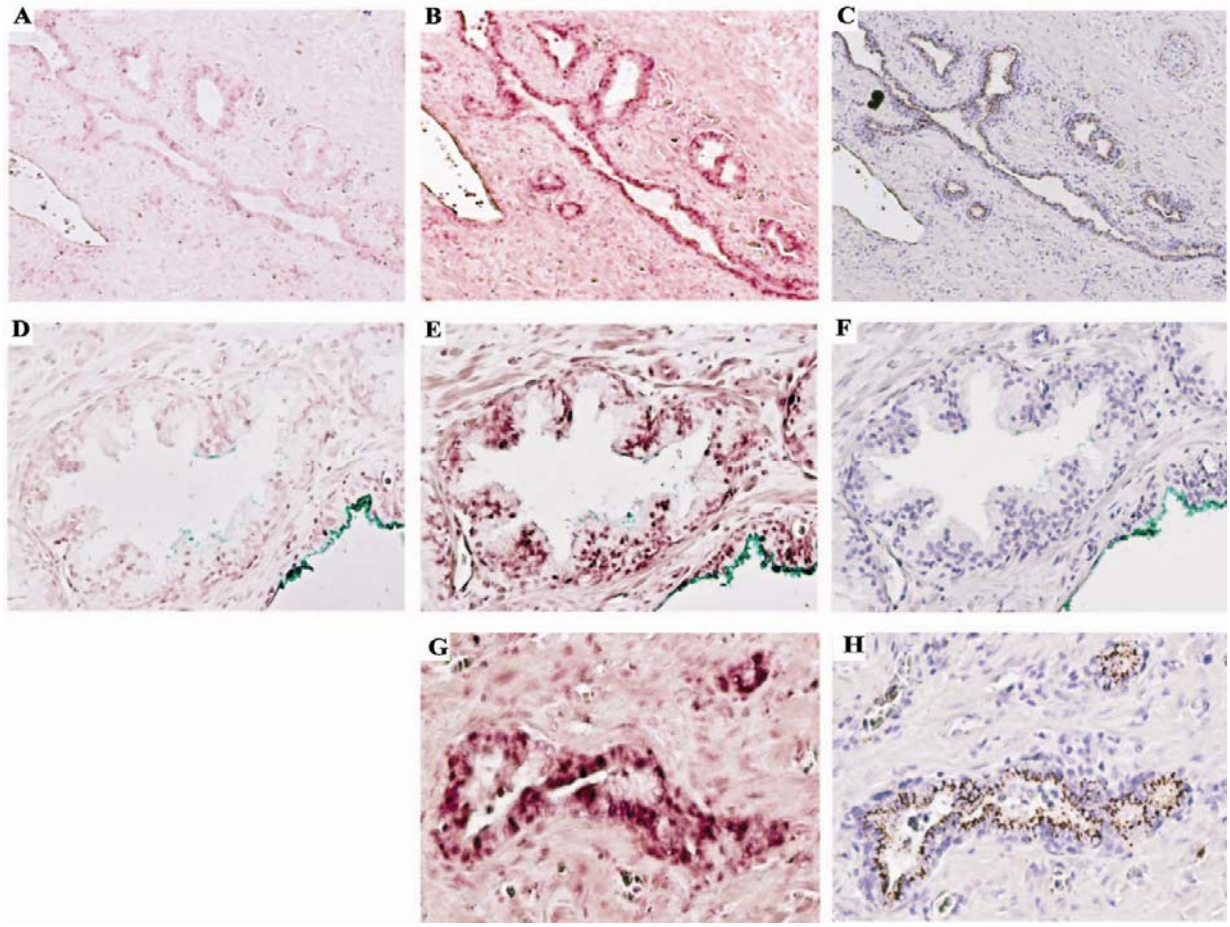
Figure 3. Laser capture microdissection of cancerous prostate. (**A-C**), an atrophic gland immunostained with anti-TAg is shown before laser capture (**A**), after the thermoplastic film is removed (**B**), and as the captured sample (**C**). (**D-F**), tumor cells immunostained with anti-p53 are

shown before laser capture (**D**), after removal of thermoplastic film (**E**) and in the captured sample (**F**). (**G**), representative chromatogram of specimen 1 from Table 3, showing comparison of the DNA sequence of exon 5, nucleotides 13075-13111, of the *p53* gene from laser captured TAg-expressing atrophic cells (bottom) and from the entire thin section (top). Arrow shows a mix of wild type (cytosine) and mutant (thymidine) form of *p53*, which corresponds to amino acid 140. Careful analysis of the chromatogram shows both nucleotides even though the software recognized that position as a cytosine. The underline represents the wild type codon for 140 at the same position from laser captured TAg expressing cells.

Figure 4. Immunohistochemistry for VP1. **A** and **B** are TAg-positive cancerous prostate tissue sections immunostained with IgG_{2a} isotype control or anti-VP1 monoclonal antibody, respectively. (**C**-**D**) are mock or BKV-infected kidney epithelial cells in culture, respectively, immunostained with anti-VP1 antibody. Magnification: x 100 (**A**-**B**); x 400 (**C**-**D**)

Figure 5. Model of induction of prostate cancer by BKV. See Discussion for details.


A
B
C
D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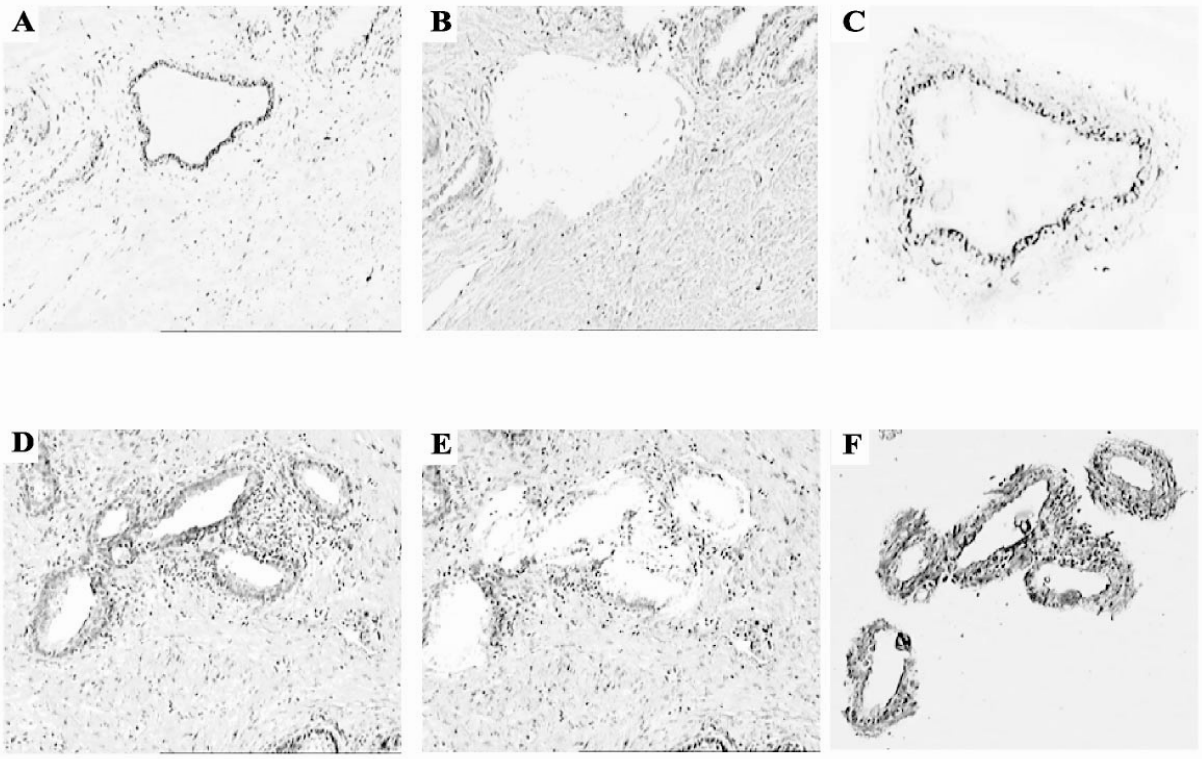
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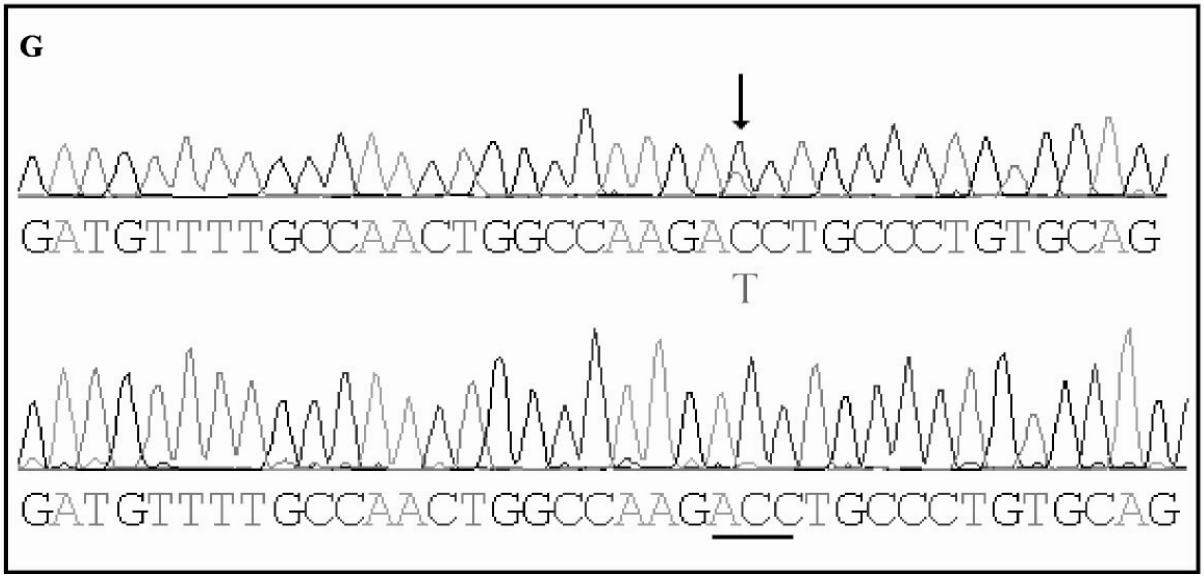
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T
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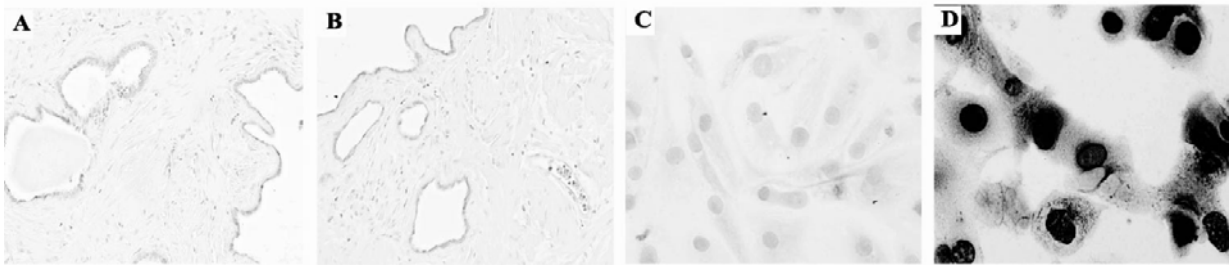
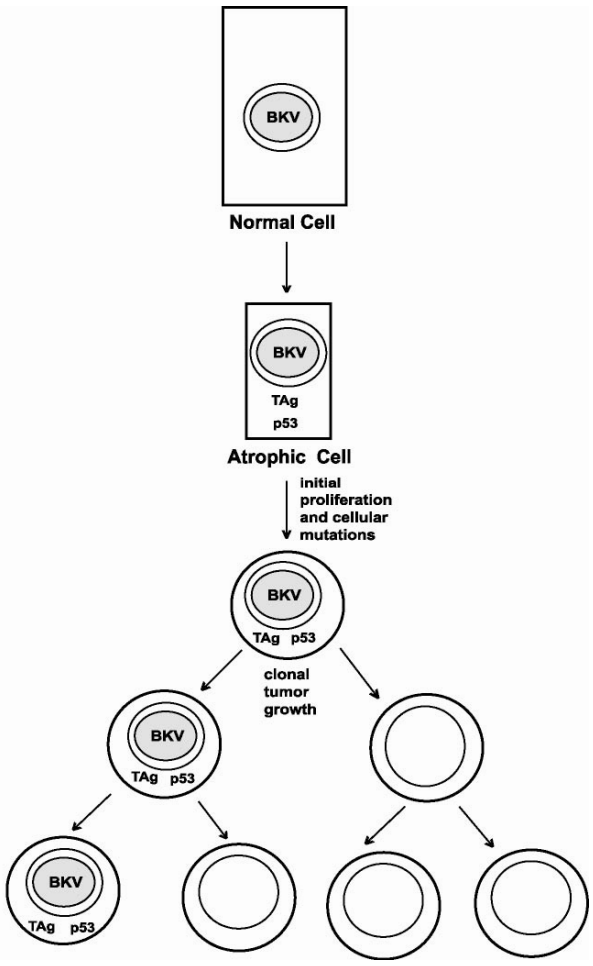

A
B
C
D


BKV
Normal Cell
BKV
TAg
p53
Atrophic Cell
initial
proliferation
and cellular
mutations
BKV
TAg p53
clonal
tumor
growth
BKV
TAg p53
BKV
TAg p53